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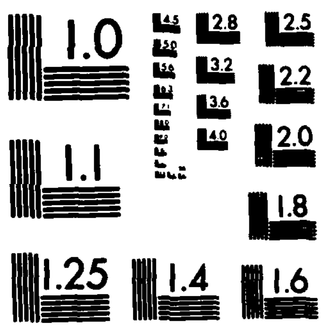

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BLOOD SUBSTITUTE: EFFECTS ON DRUG PHARMACOKINETICS

ANNUAL SUMMARY REPORT

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The effect of exchange transfusion with Fluosol DA 20% (FDA) or stroma-free hemoglobin (SFH) on the pharmacokinetics of penicillin and diazepam and on the effectiveness of penicillin therapy of pneumococcal infection in rats was determined. FDA prolongs the half-life of penicillin and does not alter the pharmacokinetics of diazepam. SFH increases the volume of distribution of both penicillin and diazepam and increases the half-life of diazepam.

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Penicillin only suppresses pneumococcal infection in FDA transfused rats but cures pneumococcal infection in SFH transfused rats.

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SUMMARY

The effect of exchange transfusion with Fluosol DA 20% (FDA) or stroma-free hemoglobin (SFH) on the pharmacokinetcs of penicillin and diazepam and on the effectiveness of penicillin therapy of pneumococcal infection in rats was determined.

Rats were sham transfused or exchange transfused with 25 ml of FDA or SFH. Within 30 minutes of transfusion, groups of five animals were given 1750 U of penicillin or 2 mg of diazepam by intravenous push injection. Serial plasma samples were obtained for determination of penicillin concentrations by bioassay or diazepam concentrations by high pressures liquid chromatography. After transfusion with FDA, the penicillin half-life (t) was prolonged ($p = 0.02$) but the volume of distribution (Vd), clearance (Cl), and area under the curve (AUC) were not different ($p \geq 0.2$) compared to sham transfused animals. Diazepam pharmacokinetics in FDA and sham transfused animals were not different ($p \geq 0.16$). After transfusion with SFH, the penicillin Vd was increased ($p = 0.03$) but t , Cl, and AUC were not different ($p \geq 0.11$) compared to sham transfused animals. Diazepam t and Vd were increased ($p \leq 0.04$) but Cl and AUC were not different ($p \geq 0.10$) compared to sham transfused animals.

Twenty-four hours after transfusion with FDA or SFH rats were challenged intraperitoneally (IP) with 10^4 to 10^5 CFU of type III pneumococcus. Twenty-two hours later, therapy was begun with penicillin G, 1750 U, or 0.5 ml of saline, intraperitoneally every 6 hours for 20 doses; all surviving rats were observed for another 6 days. All FDA transfused, uninfected rats ($n = 5$) lived for the 281 hours test period. All FDA transfused, infected, saline treated rats ($n = 8$) died by 73.5 hours after challenge; all sham transfused, infcted, saline treated rats ($n = 6$), except one, died by 61.5 hours after challenge ($p = 0.74$). Twelve of 15 (80%) of the sham transfused, infected, penicillin treated rats were alive at 281 hours; only 3 of 13 (23%) of the FDA transfused, infected, penicillin treated rats were alive at 281 hours ($p = 0.005$). Of the 13 penicillin treated rats from both infected, penicillin treated groups that died, 12 died after therapy was stopped. Pneumococcus was found in peritoneal fluid, pleural fluid, and/or blood cultures from 8 of 10 rats that died and were examined. All SFH transfused, uninfected rats ($n = 5$) lived for the 281 hour test period. All SFH transfussed, infected, saline treated rats ($n = 5$) died by 55 hours after chhallenged. All sham transfused, infected, saline treated rats ($n = 10$) died by 37 hours after challenge ($p = 0.43$). Seven of 10 (70%) of the sham transfused, infected, penicillin treated rats were alive at 281 hours. Nine of 10 (90%) of the SFH transfused, infected, penicillin treated rats were alive at 281 hours ($p = 0.24$). Of the four penicillin treated rats from both infected, penicillin treated groups that died, all died within 27 hours of the start of penicillin therapy. Pneumococcus was found in the peritoneal fluid, pleural fluid, and/or blood cultures of two of the four that died.

These data suggest that: (1) FDA and SFH do alter the disposition of penicillin and diazepam in vivo; (2) FDA may block renal tubular secretion of penicillin, the major rate limiting step in penicillin elimination; (3) the increase in Vd associated with SFH may be due to decreased intravascular binding; (4) FDA decreases the effectiveness of penicillin therapy of pneumococcal infection; and (5) SFH does not alter the effectiveness of penicillin therapy of pneumococcal infection.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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VI. Body of Report

A. Introduction

Oxygen-carrying blood substitutes include a variety of substances such as buffers (1-3), plasma (4,5), perfluorocarbon emulsions (5-9), and stroma-free hemoglobin (10-14). Of these, perfluorocarbon emulsions and stroma-free hemoglobin (SFH) have been the most extensively studied (5,14). One perfluorocarbon emulsion, Fluosol-DA 20% (FDA; Alpha Therapeutics, Los Angeles, CA) has shown enough promise that clinical trials have been initiated (15).

The major use of the blood substitutes will be to treat refractory anemias, blood loss, and vascular occlusions, and for organ perfusion and preservation (16). An important use of the blood substitutes will be to resuscitate trauma victims while waiting for blood or blood products to reach them. Patients, who receive blood substitutes, will usually receive other drugs, such as antibiotics, analgesics, and sedatives. When coadministered with a blood substitute, the pharmacokinetics of these other drugs may be altered to such an extent that they are toxic or are ineffective.

Studies were undertaken to determine the pharmacokinetcs of penicillin, diazepam, morphine, antipyrine, and sulfamethazine after transfusion of FDA and SFH in rats. The effectiveness of penicillin for the treatment of pneumococcal infection in rats after transfusion with FDA and SFH was also studied.

The results of penicillin and diazepam pharmacokinetics and the effectiveness of penicillin therapy are completed and are reported in this communication.

B. Materials and Methods

Animals. Young, adult, male Sprague-Dawley rats weighing 250 to 350 g obtained from Sasco, Omaha, NE, were used for these studies.

Transfusion of FDA. After being anesthetized with methoxyfluorane, the left external jugular vein of the rat was exposed. After the left external jugular vein was isolated, a plastic cannula was inserted cephalad and anchored with a suture. A 25 gauge scalp vein needle was inserted into a lateral tail vein. The cannula and needle were kept patent with a heparin solution (25 units/ml). An isovolumetric exchange transfusion was then done by infusing 25 ml of FDA or SFH into the tail vein by infusion pump while simultaneously withdrawing fluid from the jugular cannula. The transfusion took 25 minutes. After completion of the transfusion, the tail vein needle was removed. The external jugular vein cannula was left in place for serial blood sampling for pharmacokinetic studies. In animals that were to be infected subsequently, the external jugular vein was ligated above and below the venotomy and the cannula was removed. The wound was closed with autoclips and the animal placed in a cage with supplemental oxygen for 24 hours.

Sham Transfusion. After being anesthetized with methoxyfluorane, the left external jugular vein of the rat waas exposed. After the left external jugular vein was isolated, a plastic cannula was inserted cephalad and anchored with a suture. The jugular vein cannula was used for serial blood sampling for pharmacokinetic studies. In animals that were to be infected subsequently, the left external vein was only ligated in two places. The wound was closed with autoclips and the animal was returned to its cage for recovery.

Penicillin Assay. The concentrations of penicillin were determined using a disk diffusion method. Blank, 6 mm antibiotic susceptibility disks were moistened by dipping the disk into the test samples or into reference standards diluted in pooled, normal rat plasma. The excess fluid was blotted off the disks. Then, they were placed on the surface of antibiotic medium A, pre-seeded with *Staphylococcus aureus* (ATCC 6538-P) in 150 mm petri dishes. All test and reference samples were tested in duplicate. Inoculated plates were incubated at 37°C for 18 hours. Zones of inhibition around the disks were measured with a vernier caliper. Antibiotic concentrations were determined by comparing the mean zone of inhibition of each sample with a curve constructed from the mean zones of inhibition around the reference standards. The sensitivity of his assay was 0.05 U/ml.

Diazepam Assay. One hundred µl of test sample, to which was added 50 µl of oxazepam (internal standard, 20 mg/L), was added to 250 µl of a saturated solution of sodium phosphate tribasic. After mixing, 1 ml of ethyl acetate was added, the sample was vortexed, and the sample was then centrifuged. The top organic layer was transferred to another tube and blown dry under nitrogen gas. The residue was taken up in 100 µl of methanol. Twenty-five µl were injected onto the high pressure liquid chromatography column. Ion-paired chromatography was done using a reverse phase C_{18} column coupled to a 254 nm ultraviolet detector. The mobile phase used was acetonitrile: water, 45:55, containing 1 gm of sodium lauryl sulphate/L and 7 ml of glacial acetic acid/L. The mobile phase flow rate through the column was 2 ml/min. The retention times were 6 minutes and 4 minutes for diazepam and oxazepam, respectively. The concentrations of diazepam were determined by comparing the peak height ratio of diazepam to oxazepam of the test samples to a curve constructed from the peak height ratio of diazepam reference standards to oxazepam. The sensitivity of this assay was 0.25 mg/L.

Organism for the Infection Studies. The organism used was *Streptococcus pneumoniae* type III (ATCC 6303). The *S. pneumoniae* was incubated on blood agar overnight at 37°C in 5% CO_2. Then, two or three colonies were inoculated into 100 ml of Todd-Hewitt broth and incubated overnight at 37°C in 5% CO_2. The organisms were washed three times in saline and suspended in saline to a final concentration of 10^4 to 10^5 CFU for inoculation into the rats.

Pharmacokinetic Studies. Within one-half hour of sham transfusion or transfusion with FDA or SFH, 1750 U of penicillin G or 2.0 mg of diazepam was administered by bolus intravenous injection to groups of five animals. Plasma samples were obtained for determinations of penicillin concentrations before and 5, 10, 15, 20, 25, 30, 60, 120, and 180 minutes after injection. Plasma samples were obtained for determination of diazepam concentrations before and 15, 30, 45, 60, 120, 180, 240, 300, and 360 minutes after injection. All samples wre stored at -70°C until assayed.

Infection Experiments. Rats were sham transfused or exchange transfused with 25 ml of FDA or SFH. Twenty-four hours later, the rats were challenged intraperitoneally (IP) with 10^4 to 10^5 colony forming units (CFU) of type III pneumococcus. Twenty-two hours later, therapy was begun with penicillin, 1750 U, or 0.5 ml of saline, IP every six hours for twenty doses. All surviving rats were observed for another six days. Peritoneal fluid, pleural fluid, and blood was obtained for culture for pneumococcus from all animals that died while receiving or after receiving penicillin therapy.

Data Analysis. Basic pharmacokinetic parameters were calculated from the intravenous plasma concentration (C) versus time (t) curves. Half-lives were

calculated by $t_{1/2} = 0.693/k$ where k is the elimination rate constant or slope of the terminal portion of log C versus t curves. Volumes of distribution were calculated by Vd = Dose/Co where Co is the concentraton at t = 0 obtained by extrapolation. For those drugs exhibiting a second compartment then Vd = Dose/ $\frac{A}{\alpha} + \frac{B}{\beta} \times \beta$ where A and B are the concentration intercepts obtained by extrapolating each exponential to t = 0, and α and β are the rate constants obtained from the slopes of the two exponentials. Clearance values were determined by Cl = k x Vd. Survival curves were constucted using the methods of Kaplan Meier (17). Comparisons between survival curves were made using the generalized Kruskal-Wallis statistic (18). The significance of other differences were determined using Fisher's exact test or Student's t-test.

C. Results

After transfusion with FDA, the $t_{1/2}$ of penicillin was increased from 11.7 ± 3.2 (mean ± standard deviation) to 18.9 ± 4.6 min ($p = 0.02$), Table 1. However, the Vd, Cl and area under the log C versus t curve (AUC) were not significantly increased. After transfusion with SFH, the Vd of penicillin was increased from 136.9 ± 93.6 to 280.3 ± 76.5 ml ($p = 0.03$), Table 1. In contrast the $t_{1/2}$, Cl and AUC were not significantly increased.

After transfusion with FDA, the pharmacokinetics of diazepam were not significantly different when compared to sham transfused animals ($p \geq 0.15$), Table 1. However, after transfusion with SFH, the $t_{1/2}$ of diazepam increased from 33.3 ± 18.5 to 60.1 ± 16.8 min ($p = 0.04$) and the Vd of diazepam increased from 291.1 ± 68.4 to 1255.1 ± 659.4 ml ($p = 0.01$), Table 1.

All FDA transfused, uninfected rats (n = 5) lived for the 281 hours test period (Fig. 1). All FDA transfused, infected, saline treated rats (n = 8) died by 73.5 hours after challenge. All sham transfused, infected, saline treated rats (n = 6), except one, died by 61.5 hours after challenge ($p = 0.74$). Twelve of 15 (80%) of the sham transfused, infected, penicillin treated rats were alive at 281 hours. Only 3 of 13 (23%) of the FDA transfused, infected, penicillin treated rats were alive at 281 hours ($p = 0.005$). Of the 13 infected, penicillin treated rats from both groups that died, 12 died after therapy was stopped. Pneumococcus was found in the peritoneal fluid, pleural fluid, and/or blood cultures of 8 of 10 rats that died and were examined (Table 2). Penicillin concentrations after IP injection immediately after transfusion with FDA were higher with sustained elevation compared to sham transfused rats (Fig. 2 and 3). However, 48 hours and 168 hours after transfusion with FDA, the concentratiions of penicillin were similar in sham transfused and FDA transfused rats.

All SFH transfused, uninfected rats (n = 5) lived for the 281 hour test period (Fig. 4). All SFH transfused, infected, saline treated rats (n = 5) died by 55 hours after challenge. All sham transfused, infected, saline treated rats (n = 10) died by 37 hours after challenge ($p = 0.43$). Seven of 10 (70%) of the sham transfused, infected, penicillin treated rats were alive at 281 hours. Nine of 10 (90%) of the SFH transfused, infected, penicillin treated rats were alive at 281 hours ($p = 0.24$). Of the four infected, penicillin treated rats from both groups that died, all died within 27 hours of the start of penicillin therapy. Pneumococcus was found in the peritoneal fluid, pleural fluid, and/or blood cultures of two of the four that died.

D. Discussion

The data suggest that FDA and SFH do alter the disposition of penicillin and diazepam in vivo. The $t_{1/2}$ of penicillin is increased after transfusion with FDA. Therapeutic agents are considered to be completely eliminated after five half-lives. Thus, in sham transfused animals, penicillin would be completely eliminated one hour after administration. However, in FDA transfused animals, penicillin would not be completely eliminated until one and one-half hours after administration. If a one hour dosing interval were used in sham transfused animals, no accumulation would take place. In conctrast, if a normal dosing interval of one hour were used in FDA transfused animals, accumulation would occur and toxic levels would be reached.

FDA did not alter the pharmacokinetics of diazepam significantly. This result could not be predicted ahead of time and suggests that the pharmacokinetics of drugs that might be coadministered with FDA are not altered by FDA in a predictable manner.

The Vd of penicillin and diazepam are increased after transfusion with SFH. And, the $t_{1/2}$ of diazepam is increased after transfusion with SFH. This means that the plasma concentration of these two drugs is lower in SFH transfused animals than in sham transfused animals. In the case of SFH transfused animals, the effect of diazepam would be prolonged after stopping the drug compared to sham transfused animals because of the prolonged $t_{1/2}$.

Prolongation of the $t_{1/2}$ of penicillin after FDA transfusion suggests that FDA may block renal tubular secretion of penicillin, the major rate limiting step in penicillin elimination (19). The increase in Vd of penicillin and diazepam with SFH may be due to decreased intravascular binding of the drugs. The relatively larger increase in Vd for diazepam is expected because penicillin is normally only 65% bound intravascularly (19) whereas diazepam is 99% bound (20).

Thus, the blood substitutes, FDA and SFH, alter drug pharmacokinetics. The type of alteration is dependent on the blood substitute and drug combination. The effect of one blood substitute-drug combination does not help predict the effect of that blood substitute on the disposition of another drug. And, the effect of one blood substitute does not help predict the effect of another blood substitute.

Penicillin was found to only suppress pneumococcal infection in FDA transfused rats and relapse of infection occurred after therapy was stopped. Failure of penicillin therapy to cure pneumococcal infection in FDA transfused rats was not caused by alteration of the plasma concentrations of IP administered penicillin. In contrast, penicillin was found to cure pneumococcal infection in SFH transfused rats. Because perfluorocarbon compounds are cleared from the circulation by phagocytosis by fixed tissue macrophages (21), it is suspected that FDA adversely effects the immunocompetence of rats. This may lead to their inability to clear the pneumococci from their bodies in spite of treatment with penicillin. SFH also is cleared from the circulation by phagocytosis but other mechanisms, such as glomerular filtration, are also operative (14). Thus, the apparent lack of effect of SFH on penicillin therapy of pneumococcal infection is most likely related to the magnitude of the effect rather than the lack of effect on the immunocompetence of rats.

E. Conclusions

1. FDA and SFH alter the disposition of penicillin and diazepam in vivo.

2. FDA may block the renal tubular secretion of penicillin.

3. SFH may increase the Vd of penicillin and diazepam by decreasing intravascular binding.

4. FDA may suppress the immunocompetence of rats to a greater extent than SFH.

F. Recommendations

The following studies should be done to futher characterize the changes found so far:

1. The duration of FDA's effect on penicillin pharmacokinetcs.

2. The effect of FDA on the renal tubular secretion of penicillin.

3. The duration of SFH's effect on penicillin and diazepam pharmacokinetics.

4. The effect of SFH on intravascular binding of penicillin and diazepam.

5. Characterization of pneumococcal infection in FDA and SFH transfused rats.

6. Characterization of immunologic responsiveness of FDA and SFH transfused rats.

TABLE 1

PHARMACOKINETICS OF PENICILLIN AND DIAZEPAM AFTER TRANSFUSION WITH FDA OR SFH IN RATS

Drug	Transfusion Group	Weight	Hematrocrit (%) Before Transfusion	Hematrocrit (%) After Transfusion	$t_{1/2}$ (min)	Vd (ml)	Cl (ml/min)	AUC[a]
Penicillin	SHAM	337.4 ± 20.8[b]	43.6 ± 1.1	N.A.[c]	11.7 ± 3.2	305.8 ± 83.5	18.6 ± 5.3	100.7 ± 29.
	FDA	342.0 ± 14.8	43.0 ± 3.4	17.8 ± 3.0	18.9 ± 4.6	372.5 ± 153.1	14.3 ± 15.4	151.4 ± 98.1
					p = 0.02	p = 0.3	p = 0.4	p = 0.2
	SHAM	302.0 ± 19.2	43.5 ± 1.7	N.A.	8.9 ± 3.2	136.9 ± 93.6	9.9 ± 3.6	200.6 ± 89.5
	FDA	314.0 ± 13.4	41.3 ± 1.5	10.3 ± 1.7	18.2 ± 11.0	280.3 ± 76.5	14.8 ± 9.6	196.0 ± 83.8
					p = 0.1	p = 0.03	p = 0.3	p = 0.96
Diazepam	SHAM	286.5 ± 13.2	N.D.[d]	N.A.	16.3 ± 7.9	444.9 ± 408.2	17.9 ± 18.3	208.1 ± 157.1
	FDA[e]	303.6 ± 9.2	44.4 ± 6.1	16.1 ± 2.1	25.8 ± 17.2	1034.6 ± 709.4	28.3 ± 15.9	93.4 ± 58.8
					p = 0.3	p = 0.15	p = 0.39	p = 0.21
	SHAM	300.0 ± 12.2	42.4 ± 1.1	N.A.	33.3 ± 18.5	291.1 ± 68.4	8.1 ± 5.5	366.3 ± 242.9
	FDA	289.0 ± 19.5	43.6 ± 2.3	12.6 ± 1.5	60.1 ± 16.8	1265.1 ± 294.9	13.8 ± 4.5	160.2 ± 59.5
					p = 0.04	p = 0.01	p = 0.11	p = 0.1

[a] The units for AUC are U/ml x min for penicillin and μg/ml x min for dizaepam.
[b] mean ± standard deviation.
[c] N.A. = not applicable.
[d] N.D. = not done.
[e] n = 4 for this group; n = 5 for all others.

TABLE 2

RESULTS OF CULTURE OF BLOOD, PLEURAL FLUID AND PERITONEAL FLUID
AT TIME OF DEMISE OF PENICILLIN TREATED ANIMALS

Transfusion Group	Total No.	No. Died	No. Cultured	No. with a Culture Positive for Pneumococc
FDA + penicillin	13	10	7	6
Sham + penicillin	15	3	3	2

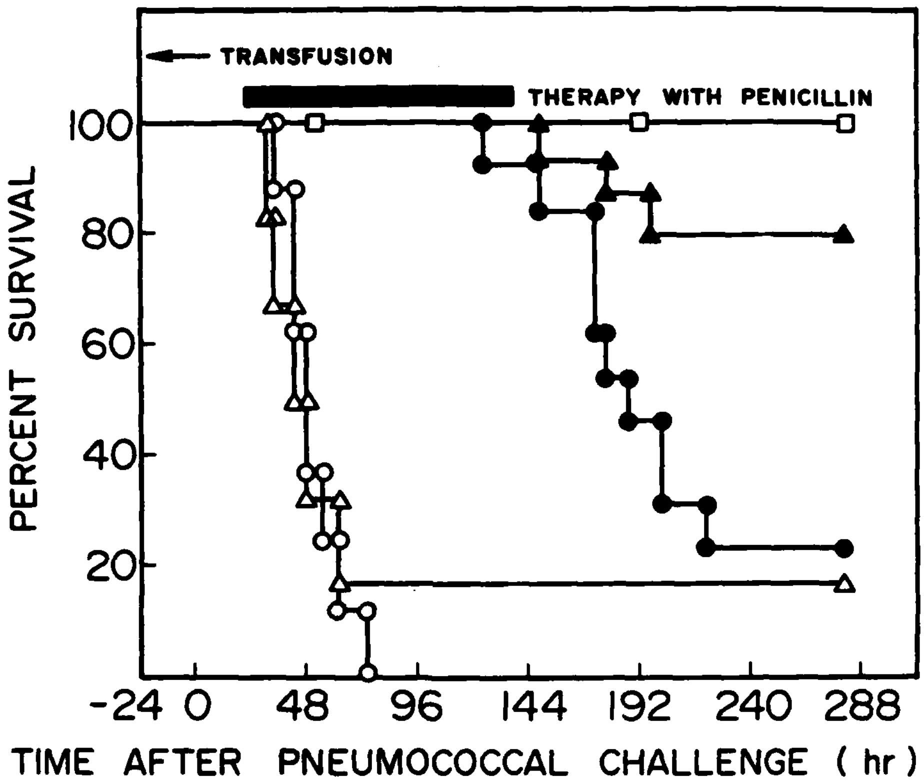

FIGURE 1. Survival of rats after transfusion with FDA (□—□); transfusion with FDA, challenge with pneumococcus, and saline treatment (O——O); transfusion with FDA, challenge with pneumococcus, and penicillin therapy (●——●); sham transfusion, challenge with pneumococcus, and saline treatment (Δ——Δ); and sham transfusion, challenge with pneumococcus, and penicillin therapy (▲——▲).

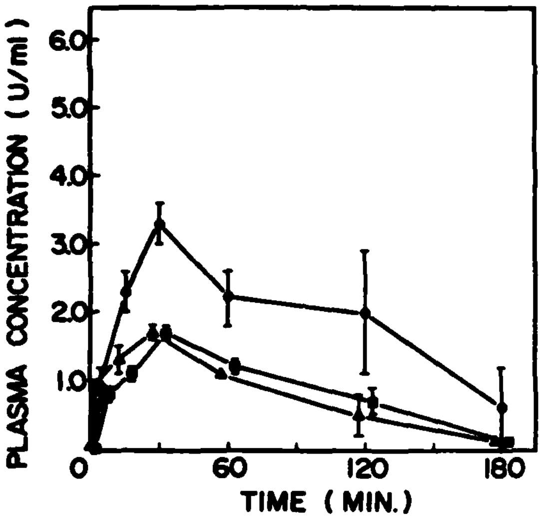

FIGURE 2. Penicillin concentrations (mean ± standard error of the mean) after IP injection in rats immediately (n = 4, ●—●), 48 h (n = 3, ▲—▲), and 168 h (n = 3, ■—■) after sham transfusion.

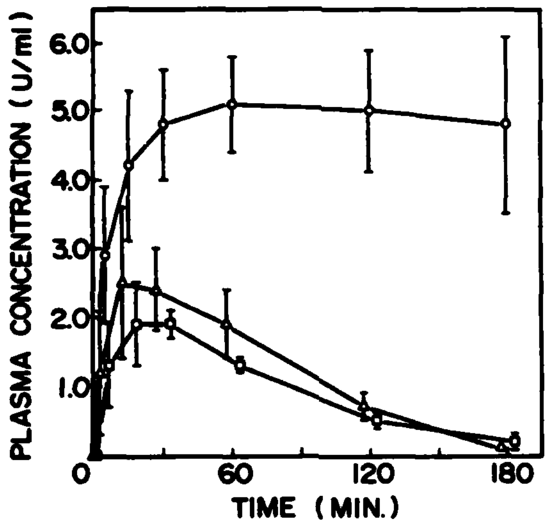

FIGURE 3. Penicillin concentrations (mean ± standard error of the mean) after IP injection in rats immediately (n = 4, O—O), 48 h (n = 3, Δ--Δ), and 168 h (n = 3, □—□) after transfusion with FDA.

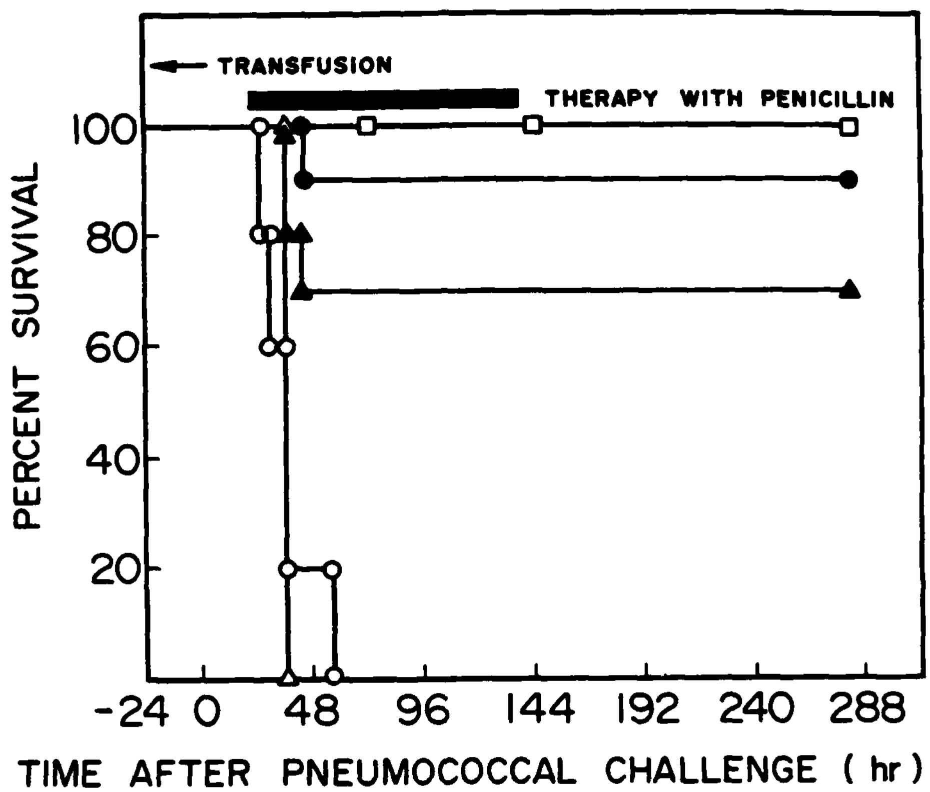

FIGURE 4. Survival of rats after transfusion with SFH (□—□); transfusion with SFH, challenge with pneumococcus, and saline treatment (O——O), transfusion with SFH, challenge with pneumococcus, and penicillin therapy (●——●); sham transfusion, challenge with pneumococcus, and saline treatment (△——△); and sham transfusion, challenge with pneumococcus, and penicillin therapy (▲——▲).

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VIII. Glossary

ATCC = American Type Culture Collection

AUC = Area Under the Curve

CFU = Colony Forming Units

Cl = Clearance

FDA = Fluosol-DA 20%

HPLC = High Pressure Liquid Chromatography

SFH = Stroma-Free Hemoglobin

$t_{1/2}$ = Half-life

Vd = Volume of Distribution

IX. Distribution List

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